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Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine

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Abstract

Results from recent serological surveys and epidemiological studies show that pigs raised in a variety of management systems can be carriers of the tissue cyst stage of *Toxoplasma gondi*. This parasite can be transmitted to humans through the consumption of improperly prepared pork, making detection and removal of infected swine carcasses from the food chain an important food safety issue. Several methods are available for detection of *T. gondii* infected swine, including serological assays, polymerase chain reaction, and animal bioassays. The aim of the present study was to compare the detection sensitivities of six of these commonly used methods for detection of *T. gondii* infection in tissues from naturally and experimentally infected pigs. The results indicate that a serum-based ELISA is the most sensitive method, of those tested, for detection of *T. gondii* infected swine.

Keywords: Swine; *Toxoplasma gondii*; Serology; PCR; Bioassay; Diagnosis

1. Introduction

Toxoplasma gondii is one of the most common parasitic infections of humans and other warm-blooded animals. It has been found worldwide from Alaska to Australia. Nearly one-third of humanity has been exposed to this parasite. In most adults it does not cause serious illness, but it can cause blindness and mental retardation in congenitally infected children, blindness in persons infected after birth, and devastating disease or death in immunocompromised individuals.

Although 20–22% of women of childbearing age in the United States have antibodies against *T. gondii* and are immune to acquiring toxoplasmosis during pregnancy, greater than 70% remain at risk (Dubey and Beattie, 1988; Lopez et al., 2000). When a previously uninfected woman acquires *T. gondii* infection during

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pregnancy, there is a 20–50% probability that her fetus will become infected. It has been estimated that 0.1% of children are infected in utero, equating to about 3300 infected children born every year in the United States. Although most infected children do not have obvious symptoms at birth, many manifest the disease later in life (i.e., loss of vision and mental retardation).

Though most postnatally-acquired infections are subclinical, devastating illness develops in patients with acquired immunodeficiency syndrome (AIDS). Other patients at risk include those given immunosuppressive therapy for organ transplants or malignancies. Economic losses associated with toxoplasmosis in humans are high, and include educational and residential care costs of mentally retarded people, as well as deaths due to encephalitis and blindness (Roberts and Frenkel, 1990; Roberts et al., 1994).

Consumption of raw or undercooked meat products containing *T. gondii* tissue cysts (bradyzoites) or ingestion of food or drink contaminated with infectious oocysts (sporozoites) from cat feces are risk factors associated with *T. gondii* infection. The relative risk to US consumers of acquiring *T. gondii* infection from undercooked meat, including pork, was recently determined in a nationwide retail meats survey. The survey of 698 retail outlets determined the prevalence of viable *T. gondii* tissue cysts in commercially available fresh pork products to be 0.38% (Dubey et al., 2005). Increased consumer awareness of the potential risks of acquiring *T. gondii* from fresh pork products represents a potentially serious problem for the US pork industry, and is a critical food safety issue.

To calculate the risk to consumers from the consumption of infected meats, and to determine the best management practices for the pork industry which will reduce or eliminate the potential of infection to animals on the farm, harmonization of accurate and sensitive testing methods is needed to identify infected animals at the farm level and to detect infected carcasses on the production line. In the present study, we have compared detection sensitivities of serologic assays (a validated commercial enzyme linked immunoabsorbent assay (ELISA) and the modified agglutination test (MAT)), and molecular assays (the polymerase chain reaction (PCR) using the TgB1 primers for both direct and semi-nested PCR, and real time PCR with primers and probes for the ITS1 region of the 18S rRNA gene), with the cat bioassay as the gold standard using tissues collected from naturally- and experimentally-infected pigs.

2. Materials and methods

2.1. Collection of tissues from naturally-infected pigs

Twenty-five pigs were purchased from a farrow-to-finish swine farm in Massachusetts (Dubey et al., 2002). Before slaughter, venous blood for serum collection was obtained from all 25 pigs, and hearts and tongues were collected for bioassay (Dubey et al., 2002).

Additional tissues (boneless pork loin or center cut chops) from naturally-infected and uninfected pigs were collected from retail meat counters during conduct of the National Retail Meats Survey (NRMS) (Dubey et al., 2005). Eight *T. gondii* positive pork samples (as determined by cat bioassay) were purchased during the survey; details of the sample selection can be found in Dubey et al. (2005). A total of 34 samples collected from retail outlets were tested in this study.

2.2. Collection of tissues from experimentally-infected pigs

Ten *T. gondii*-seronegative pigs (~50 kg each, 5 months of age, Ernst Farms, Clear Spring, MD) were infected per o.s. with 1000 sporulated *T. gondii* VEG strain oocysts (Dubey et al., 1996). Pigs were euthanized at 60 days post-infection, and both loins were removed from each animal.

2.3. Cat bioassay of naturally infected pig tissues

All cats used in the present study were 10–12 weeks old, raised in isolation from birth (Liberty Research, Waverly, NY), and had not been fed uncooked meat before these experiments. Blood was drawn from a jugular vein of each cat 1 week before feeding pig tissues, and the sera were tested for antibodies to *T. gondii* using the MAT as described by Dubey and Desmonts (1987). No demonstrable MAT antibodies were found in a 1:25 dilution of the sera.

After trimming fat, connective tissue, and epithelia, 100–250 g of myocardium and tongue from each naturally-infected pig from the Massachusetts farm were fed individually to 25 cats over a 2–3-day period. Each cat was housed individually and the feces were collected daily for 14 days beginning 3 days after feeding of meat. Feces were microscopically examined for oocysts by fecal flotation (Dubey, 1995).

Boneless pork loin or center cut pork chops collected during the NRMS were trimmed of fat and a 100 g portion was collected from each sample for bioassay in cats. The 100 g portions from six individual pork samples were pooled and 600 g of meat was fed to one cat over a period of 2–3 days. The cats were treated as described above, and feces were examined for oocysts for 14 days as described.

2.4. Cat bioassay of experimentally infected pig tissues

A 50 g sample was removed from the loin muscle of each of 10 experimentally infected pigs and fed to individual cats as above; feces were collected and examined for oocysts as described above.

2.5. Serum ELISA

Serum antibodies to *T. gondii* were determined in the pigs by ELISA using a validated commercial ELISA kit (Safepath Laboratory, Carlsbad, CA). Serum were tested at a 1:50 serum dilution on the day of infection and at the time of euthanasia (60 days PI). The ELISA kit uses formalin-fixed whole tachyzoites as antigen, and has been validated for use with pork samples (Gamble et al., 2005). Reference positive and negative controls were established using a pool of five *T. gondii* positive (P1) and a pool of five *T. gondii* negative (P2) swine serum samples. ELISA ODs were determined for 20 replicates of P1 and P2, and a mean OD was determined for each (P1r and P2r). P1 and P2 were included on each plate, and a corrected OD value was calculated for each sample using the formula as described by Lind et al. (1997): corrected OD = (OD sample − ODP2) × ODP1r/ODP1 + ODP2r. A positive cut-off was established as ODP2r + three times the standard deviation from ODP2r. Plates were read at 405 nm using a V_{max} ELISA reader (Molecular Devices, Sunnyvale, CA). Positive (PPV) and negative predictive values (NPV) were calculated (Greiner and Gardner, 2000a,b), where PV = number of correctly classified samples/number of truly diseased or non-diseased samples.

2.6. Tissue fluid ELISA

Tissue fluids, for use in the ELISA test, were collected from individual 10 g samples from the diaphragm of naturally- and experimentally-infected animals at slaughter, or 50 g samples from boneless pork loin or pork loin chops purchased during the NRMS. Samples were frozen at −18 °C overnight, then thawed at room temperature for fluid collection. Tissue fluids were cleaned of debris by centrifugation. The ELISA was performed using the commercial test kit described above using tissue fluids at a 1:10 dilution. Reference positive and negative tissue fluid controls were established using a pool of five *T. gondii* positive (P1) and a pool of five *T. gondii* negative (P2) swine tissue fluid samples and were used as described above for serum samples. A positive cut-off was established as ODP2r + three times the standard deviation from ODP2r.

2.7. MAT serology

All pig sera were initially screened using dilutions of 1:25, 1:50, 1:100, and 1:500. Serum samples from pigs that were negative in the initial MAT screen were further tested, starting at a 1:10 serum dilution. Negative control sera were obtained from five *T. gondii*-negative pigs from the swine production herd at the Beltsville Agricultural Research Center.

2.8. PCR using the TgB1 primers (direct and semi-nested)

DNA was extracted from 0.5–1 g samples of pig tissue from each naturally- or experimentally-infected animal (heart, tongue, and loin) and from retail pork samples from the NRMS (boneless loin or center cut chops) as previously described (Sreekumar et al., 2003) using DNAzol and following the manufacturers instructions. Extracted DNA was ethanol precipitated, resuspended in TE buffer, and frozen until used. Reference DNA for both direct and semi-nested PCR was extracted from *T. gondii* VEG strain tissue cysts isolated from the brains of *T. gondii* infected Swiss-Webster mice (Taconic, Germantown, NY). Strict precautions (new plastic test tubes, clean pipets, sterile RNA/DNA-free filtered pipet tips, new reagents from separate aliquots) were used to avoid contamination of experimental samples by exogenous DNA or amplicons. PCR was performed using direct and semi-nested methods to amplify segments of the 35-fold repetitive DNA region B1 of *T. gondii* essentially as described by Lin et al. (2000). Direct PCR was performed on individual samples using the primer pair forward: 5′-GGAACTGCATCCGTT-CATGAG-3′ and reverse: 5′-TCTTTAAAGCGTT CGTGGTC-3′. Semi-nested PCR was performed using 1 μl of the amplicons resulting from the first round direct PCR as the target DNA, the forward primer 5′-TGCATAGGTTGCAGTCACTG-3′, and the reverse primer from the first round PCR. Cycling conditions for both the direct and semi-nested PCR were denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. Products from the direct and semi-nested PCR were analyzed on pre-poured 2.0% agarose E-gels along with Hae III DNA markers (Invitrogen, Carlsbad, CA).

2.9. Detection of *T. gondii* DNA by real time quantitative PCR (TaqMan)

Real time PCR was based on the procedures describes in Jauregui et al. (2001). Briefly, assays were performed on DNA prepared as described above using primers and probes for the ITS1 region of the 18S rRNA gene for *T. gondii* and universal 18S primers as positive amplification controls. Fluorescence signals measured during amplification on an ABI 7700 Sequence Detector System (TaqMan, Applied Biosystems, Foster City, CA) were processed post-amplification and were regarded as positive if the fluorescence intensity was >20-fold the standard deviation of the baseline fluorescence. This level is defined as the C_t. All the data acquisition and data analyses were performed with Sequence Detector Software (Applied Biosystems) and C_t values were recorded for statistical analysis on Excel spreadsheets.

Table 1
Assay comparisons from 10 experimentally infected pigs

Pig number	Cat bioassay; oocysts shed	Serum ELISA[a]	Tissue fluid ELISA	MAT	Direct PCR/semi-nested PCR (loin)[b]	Real time PCR (loin)
3064	Yes	1.058	0.749	>7200	−/−	−
3047	Yes	0.904	0.342	>7200	−/−	−
3085	Yes	0.812	0.291	>7200	−/+	+
3082	Yes	0.904	0.647	>7200	−/−	−/?[a]
3035	Yes	1.051	0.814	>7200	−/−	−
3027	Yes	0.963	0.632	>7200	−/−	+
3076	Yes	0.802	0.274	>7200	−/−	−
6011	Yes	1.089	0.815	>7200	−/−	−
3034	Yes	0.430	0.070	>7200	−/−	+
3016	Yes	0.553	0.188	>7200	−/−	−
Positive control, $n = 5$		$x = 0.808$	$x = 0.425$	>7200	+/+	
Negative control, $n = 5$		$x = 0.016$, S.D. = 0.030	$x = 0.013$, S.D. = 0.019	<1:10	−/−	
Positive cutoff		0.106	0.070	>1:25		

MAT: modified agglutination test; ELISA: enzyme linked immunosorbent assay, ELISA values reported are optical density (OD); x: mean; S.D.: standard deviation.

[a] Sera diluted 1:50; tissue fluids diluted 1:10.

[b] All PCR samples were negative (by agarose ethidium bromide visualization) on direct first round PCR; −/?, C_t value borderline negative.

3. Results

A total of 69 pork samples were tested by cat bioassay, direct PCR, semi-nested PCR, real time PCR, ELISA (35 sera and 69 tissue fluid samples), and MAT (35 sera and three tissue fluid samples). The results of these detection assays are shown in Tables 1–4. Thirty-nine *T. gondii* positive samples were detected in the cat bioassay out of 69 samples tested.

Using the cat bioassay as the gold standard (i.e., assuming the cat bioassay detected 100% of positive samples), the detection methods ranked as follows in descending order of sensitivity: serum ELISA (test sensitivity 100%) > serum MAT (80.64%) > tissue fluid ELISA (76.9%) > real time PCR (20.51%) > semi-nested PCR (12.82%) > direct PCR (0%).

4. Discussion

In this study, we compared the sensitivities of six methods for detection of *T. gondii* in naturally and experimentally infected swine. Previous studies have demonstrated that the cat bioassay is a highly sensitive method of detection (Dubey and Frenkel, 1976; Dubey, 2001). It is considered the "gold standard" of detection methods since cats become infected with very low numbers of parasites; therefore all measures

Table 2
Assay comparisons from 25 naturally infected pigs

Pig number	Cat bioassay; oocysts shed	Serum ELISA[a]	Tissue fluid ELISA	MAT	Semi-nested PCR (heart/tongue)[b]	Real time PCR (heart/tongue)
1	No	0.081	0.171	<1:10	−/−	−/−
2	Yes	0.742	0.326	1:40	+/+	−/+
3	Yes	1.595	0.273	≥1:100	−/−	−/−
4	Yes	0.587	0.188	1:10	−/−	−/−
5	Yes	0.837	0.436	≥1:100	−/−	−/+
6	Yes	1.274	0.095	1:40	−/−	−/−
7	Yes	0.401	0.118	1:20	−/−	−/−
8	Yes	0.190	0.083	<1:10	−/−	−/−
9	No	0.254	0.016	<1:10	−/−	−/−
10	Yes	0.851	0.247	≥1:100	−/−	−/?
11	Yes	1.150	0.207	≥1:100	−/+	+/−
12	No	0.097	0.116	<1:10	−/−	−/−
13	No	0.110	0.135	<1:10	−/−	−/−
14	Yes	0.888	0.185	≥1:100	−/−	−/−
15	Yes	0.995	0.112	1:20	−/−	−/−
16	Yes	1.407	0.171	1:20	−/−	−/−
17	Yes	1.718	0.150	<1:10	−/−	−/−
18	Yes	1.044	0.097	≥1:100	−/−	−/−
19	Yes	0.933	0.171	≥1:100	+/+	−/−
20	Yes	0.641	0.102	≥1:100	+/−	−/−
21	Yes	1.003	0.059	≥1:100	−/−	+/−
22	Yes	0.815	0.066	≥1:100	−/−	−/+
23	Yes	0.587	0.070	≥1:100	−/−	−/−
24	Yes	0.407	0.080	≥1:100	−/−	−/−
25	Yes	0.467	0.064	≥1:100	−/−	−/−
Positive control, $n = 5$		$x = 0.808$	$x = 0.425$	>7200	+/+	+/+
Negative control, $n = 5$		$x = 0.016$, S.D. = 0.030	$x = 0.013$, S.D. = 0.019	<1:10	−/−	−/−
Positive cutoff		0.106	0.070	>1:25		

[a] Sera diluted 1:50, tissue fluids diluted 1:10, ELISA values reported are optical density (OD).
[b] All PCR samples were negative (by agarose gel ethidium bromide visualization) on direct first round PCR, only results from semi-nested PCR shown; −/?, C_t value borderline negative.

Table 3
Assay comparisons from 34 pork samples collected during the NRMS

Sample number	Cat bioassay; oocysts shed	Tissue fluid ELISA[a]	Tissue fluid MAT	Direct PCR/semi-nested PCR (loin)	Real time PCR (loin)
388	Yes	0.136	1:10	–	–
390	Yes	0.222	1:10	–	–
784	Yes	0.316	1:10	–	–
549	Yes	0.109	ND	–	–
1243	Yes	0.032	ND	–	–
1255	Yes	0.045	ND	–	–
1336	Yes	0.012	ND	–	–
1498	Yes	0.029	ND	–	–
1–13	No	0.008–0.046	ND	–	–
14–26	No	0.011–0.049	ND	–	–
Positive control, $n = 5$		$x = 0.425$		+	+
Negative control, $n = 5$		$x = 0.013$, S.D. = 0.019	<1:10	–	–
Positive cutoff		0.070	>1:25		

[a] Serum ELISA and MAT not done; serum could not be collected from retail samples. Tissue fluids diluted 1:10; ND, assay not done.

of sensitivity and specificity are dependent upon the accuracy of this method.

Most previous studies using ELISA to detect *T. gondii*-specific antibodies have examined samples obtained from experimentally infected pigs given large numbers of infective oocysts in a single dose (Waltman et al., 1984; Lind et al., 1997; Wingstrand et al., 1997). These experimental conditions presumably resulted in large numbers of tissue cysts and high titers of anti-*T. gondii* antibodies in serum; consequently good results were obtained in terms of sensitivity and specificity. This observation is borne out in the present study, in which the sensitivity of all detection methods tested was better in tissues collected from experimentally-infected animals than in tissues from naturally-infected animals. These disparate results may reflect a lower level infection in naturally infected pigs. However, the sensitivity of the serum-based ELISA was 100% in both experimentally and naturally infected pigs, suggesting that antibody response may be independent of parasite burden. A similar observation has been made in pigs infected with various doses of *Trichinella spiralis* (Murrell et al., 1985).

A previous study (Dubey et al., 1995), using naturally infected sows, reported a higher sensitivity with the MAT as compared with an ELISA. In the current study we found the serum ELISA to be superior to the MAT (100% versus 80%), since six cat bioassay positive samples were negative in the serum MAT. These findings may reflect the refinement and increased sensitivity of the serum ELISA in the recently validated test used in this study (Gamble

Table 4
Positive (PPV) and negative predictive values (NPV) for the MAT, ELISA, and PCR

Sample		MAT	Tissue fluid ELISA	Serum ELISA	Semi-nested PCR	Real time PCR
Experimentally infected pigs ($n = 10$)	PPV	100.00	90.00	100.00	10.00	30.00
	NPV	ND	ND	ND	ND	ND
Naturally infected pigs ($n = 25$)	PPV	71.43	80.95	100.00	19.00	23.81
	NPV	100.00	25.00	50.00	100.00	100.00
Retail meat samples ($n = 34$)	PPV	0.0[a]	50.00	NA	0.0	0.0
	NPV	100	100	NA	100	100

[a] MAT completed with tissue fluids instead of serum; NA, assay not done since no serum was available; ND, NPV not calculated since no negative samples were tested.

et al., 2005), however it must be noted that the four cat bioassay negative samples were identified as negative by the serum MAT, whereas two of the four cat bioassay negative samples were identified as positive by the serum ELISA, suggesting a higher rate of false positive results with this test.

The results presented here suggest that the sensitivity of the *T. gondii* ELISA using tissue fluids is considerably less than obtained when testing serum samples (76.9% versus 100%). Using the ELISA with tissue fluids, nine of 39 cat bioassay positive samples from naturally-or experimentally-infected pigs were identified as negative, and three of 30 negative samples were identified as positive. Therefore, estimating the prevalence of *T. gondii* infection in swine based on an ELISA test using tissue fluids may not be a reliable methodology, as estimates derived from this method are likely to be considerably lower than the true prevalence; further this test may result in false positives. Three of the eight tissue fluid samples from naturally infected pigs from the NRMS were also tested by MAT. These samples were negative in the MAT, though the cat bioassay indicated that they were positive; however, additional tissue fluid samples need to be tested by MAT before drawing final conclusions.

Based on the results of this study, the ELISA test performs better than the MAT for detecting serum antibodies to *Toxoplasma* in pigs. Neither test, however, can reliably detect antibody in tissue fluids collected from frozen and thawed muscle samples. Previous studies have shown that the test sensitivity of the tissue fluid ELISA is 60% (Gamble et al., 2005). Though the tissue fluid ELISA performed better (76.9% sensitivity) in the current study, the reliability problem with the tissue fluid ELISA and MAT must be taken into account when designing detection methodologies for application in slaughter facilities, since tissue fluids from whole carcasses are likely to be the source of testing materials for any serological assay performed at the plants.

The length of time needed to perform the MAT and the subjective nature of test result interpretations may render the MAT impractical for widespread application at slaughter or for epidemiological surveys performed at different labs. The serum ELISA appears to be a more useful test for the routine screening of pigs on the farm or at slaughter facilities. However, neither the serum MAT nor the serum ELISA would be suitable for individual carcass testing for purposes of assuring food safety unless individual sera can be acquired from animals before or at slaughter and results could be traced back to specific carcasses.

Although a number of reports in recent years have suggested that PCR may be a sensitive method for detection of *T. gondii* DNA in clinical settings and for epidemiological surveys (Gross et al., 1992; Wastling et al., 1993; Guy et al., 1996; Pujol-Rique et al., 1999; Jauregui et al., 2001; Contini et al., 2005), our results clearly demonstrate that these methods are the least reliable of those tested for detecting *T. gondii* DNA in swine tissues. Of the 39 known positive samples tested by the three PCR methods, only eight samples were detected by real time PCR, five positive samples were detected by semi-nested PCR, and none were detected by direct PCR. It is likely that the low reliability of the molecular assays results from the limited sample size, random distribution of tissue cysts, and perhaps low numbers of *T. gondii* tissue cysts in the swine tissues tested. As a result of these sampling issues, a negative result in the PCR assay could never be used to declare an individual pork carcass *Toxoplasma*-free. Since it is impractical to extract DNA from large quantities of pork tissue for PCR, and there is no predilection site for *T. gondii* in swine tissue that is more likely to contain tissue cysts (Dubey et al., 1986), it is unlikely that the sensitivity of the PCR assays tested here can be improved to make them useful for widespread usage.

5. Conclusion

Toxoplasmosis is a major public health issue, and, based on recent studies (Dubey et al., 2005), continues to pose a risk to public health due to its presence in pork. Demands of consumers for pathogen-free meat products have focused the attention of government regulators and the meat industry on food safety, and the necessity to produce meat that is wholesome, safe and of high quality. Standardization of current methods for the detection of *T. gondii* in live animals or animal carcasses could have an impact on assuring a safe food supply by reducing the risk associated with ingestion of fresh meats. The present study demonstrates that of the methods tested, the serum-based ELISA is the most sensitive for detection of *T. gondii* infected swine; however, additional work is urgently needed for development of practical methods for detecting *T. gondii* infected carcasses at slaughter.

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